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Plasma Steroid Hormone Profiles during HCG Induced Ovulation in Female Walking Catfish *Clarias batrachus*

Muhammad Zairin, Jr.[1], Kiyoshi Asahina[2], Kiyoshi Furukawa[1] and Katsumi Aida*[1]

[1]*Department of Fisheries, Faculty of Agriculture, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan* [2]*College of Agriculture and Veterinary Medicine, Nihon University, Setagaya-ku, Tokyo 154*

ABSTRACT—The present experiment was performed in order to investigate the response of walking catfish to human chorionic gonadotropin (HCG) and the resultant hormonal changes during ovulation. Mature female walking catfish were given a single intramuscular injection of 0.8 IU HCG/g body weight. Of 14 fish treated with a single injection of HCG, 5 fish ovulated at 20 hr and 9 fish at 24 hr following treatment. Germinal vesicle breakdown occurred after an elapse of 12–16 hr. After HCG injection, plasma testosterone peaked at 4 hr, and then gradually decreased to initial levels at 24 hr. Progesterone levels started to increase at 4 hr, and exhibited a small peak at 12 hr. Plasma 17α-hydroxyprogesterone levels began to increase at 8 hr, peaked at 12 hr, and returned to basal levels at 20 hr. Plasma 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-P)levels suddenly increased and peaked at 12 hr following treatment. 17α,20β,21-trihydroxy-4-pregnen-3-one (20β-S) also increased at 12 hr and peaked at 16 hr. Meanwhile, 17α,20α-dihydroxy-4-pregnen-3-one, a stereoisomer of 17α,20β-P, started to increase at 4 hr, reaching a peak at 12 hr, with maximum levels lower than those of 17α,20β-P or 20β-S. These peaks were concomitant to the first occurrence of GVBD. Plasma estradiol-17β levels in HCG-treated fish remained constant throughout the experiment, whereas levels in the control group were seen to decrease. These results indicate that HCG is effective in inducing ovulation in walking catfish and suggest that 17α,20β-P and/or 20β-S are the maturation inducing steroid(s) in this species.

INTRODUCTION

Changes in plasma gonadotropin (GtH) and/or steroid hormone levels during ovulation have been investigated intensively in several teleost species in order to understand the endocrine control of ovulation in fish. These investigations showed that the process of ovulation occurred following an increase of internal GtH secretion from the pituitary gland. This GtH mediates the process of final oocyte maturation and ovulation by inducing the synthesis of the maturation inducing steroid (MIS), 17α,20β-dihydroxy-4-pregnen-3-one (17α, 20β-P), in ovarian follicles [1, 2]. It has been proposed recently that 17α,20β,21-trihydroxy-4-pregnen-3-one (20β-S) is also an MIS in the Atlantic croaker [3] and the spotted seatrout [4]. 17α,20α-dihydroxy-4-pregnen-3-one (17α,20α-P) is reported to be produced during ovulation in flatfish [5]. At present, however, available information concerns a limited number of species, and information on tropical fishes is insufficient.

The walking catfish, *Clarias batrachus*, is a tropical freshwater fish belonging to the order Siluriformes. In the natural habitat of this species, spawning occurs during the rainy season and can be induced by manipulating the water level of the culture pond for practical purposes. Normal ovulation in walking catfish can be induced not only by environmental means, but also by artificial stimulation such as HCG injection [6]. However, equivalent information on hormonal changes during ovulation in *Clarias batrachus* is not yet available. Therefore, in this investigation hormonal changes during ovulation were studied using fish treated with HCG. Changes in plasma testoster-

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* To whom reprint requests should be addressed

one, progesterone, 17α-hydroxyprogesterone (17α-P), 17α,20β-P, 20β-S, 17α,20α-P and estradiol-17β were monitored during ovulation, and simultaneously, the timing of germinal vesicle breakdown (GVBD) was examined.

MATERIALS AND METHODS

Fish stock

Two-months old walking catfish were transported from Bogor, Indonesia, to Japan in 1988, and reared in an indoor concrete pond (width$\times$length$\times$depth$=1.5\times3\times0.5$ m) supplied with running freshwater (23–25°C) of deep-well origin at the Fisheries Laboratory, the University of Tokyo, Maisaka, Shizuoka Prefecture. During this rearing period, fish were subjected to a photoperiodic cycle of 12L12D. Fish were fed twice per day with commercially available trout pellets at a daily ration of 2–3% of body weight. Under the above stocking conditions, fish began to mature at an age of 9 months and thereafter, conditions of maturity were maintained without undergoing natural spawning (Zairin *et al.*, unpublished data).

Experimental fish

Twenty four 18-months old mature female walking catfish (325–675 g in weight and 34–40 cm in total length) were selected from the stock pond, and then randomly assigned to two groups, HCG-injected and control groups. Each group was further divided into two sub-groups. Each sub-group was kept separately in an indoor concrete tank (width$\times$length$\times$depth$=1\times3\times0.5$ m) supplied with running fresh water of deep-well origin 23–25°C. Photoperiod was maintained 12L12D. Fish did not receive feed during the experiment.

Treatment

HCG was purchased from Teikoku Zoki Pharmaceutical Company, Japan. In order to meet the required dosage, the original hormone was diluted with 0.6% saline solution into a 1 IU/μl solution.

The HCG-treated group received an intramuscular injection of 0.8 IU HCG/g body weight, whereas the control group received an equal volume of saline injection, just below the front edge of the dorsal fin. A single injection was given to each fish at 0800 hr.

Blood and oocyte sampling

Initial blood and oocyte samples were taken from all fish before administering the hormone solution or saline. Thereafter, sampling was carried out as follows. Up until 24 hr of post-treatment, sampling was performed alternately between the two HCG sub-groups as well as between the control sub-groups. The first sub-groups were sampled at 4, 12, 20, 24, 28 and 52 hr, and the second sub-groups were sampled at 8, 16, 24, 28 and 54 hr. Data from two sub-groups were combined in order to obtain 4 hour interval data for each type of treatment.

Approximately 0.8 ml of blood was drawn from the caudal vasculature with a heparinized syringe fitted with a 24-gauge, 1-inch needle after anesthetizing fish with 600 ppm of 2-phenoxyethanol (Wako, Japan). Blood samples were centrifuged at 3000 rpm, and plasma was stored in 1.5 ml polypropylene centrifuge tubes at -20°C until analysis by enzyme immunoassay (EIA, for 20β-S) or radioimmunoassay (RIA, for other steroids).

Following blood sampling, a small amount of oocytes was drawn by using a polyethylene cannula (2.0 mm in inner diameter, 2.5 mm in outer diameter). Oocytes were treated with clearing solution (ethanol : formalin : acetic acid$=6:3:1$) for ascertaining whether GVBD had occurred.

RIA

Steroid extraction from 0.25 ml plasma was carried out twice using 2 ml diethylether. The ether was evaporated using a centrifugal evaporator at room temperature. Samples were reconstituted with 0.5 ml of PBS containing 0.1% gelatin, 10 mM phosphate buffer and 140 mM NaCl (pH 7.5).

In this experiment, plasma testosterone, progesterone, 17α-P, 17α,20β-P, 17α,20α-P and estradiol-17β were determined by RIA. Details for RIAs for each steroid have been described previously [7–10].

Testosterone was determined using [1,2,6,7-^{3}H] testosterone (Amersham, England) and an antiserum against testosterone-11α-succinate-BSA. The antiserum was kindly provided by Prof. M.

Honma, Laboratory of Veterinary Physiology, the University of Tokyo, Japan. This antiserum against testosterone cross-reacted with 11-ketotestosterone, 5α-dihydrotestosterone, androstenedione, and androstenediol at 1.5, 30, 1.0, and 0.25%, respectively.

Progesterone was determined using [1,2,6,7-^{3}H] progesterone purchased from New England Nuclear, England, and an antiserum against progesterone-3-carboxy-methyl-oxime-BSA (Teikoku Zoki Pharm. Co., Tokyo). This antiserum cross-reacted with 17α-P, 20α-hydroxyprogesterone, pregnenolone, 11-deoxycorticosterone at 0.89, 6.73, 1.46, and 6.60%, respectively.

17α-P was determined using [1,2,6,7-^{3}H] 17α-P (New England Nuclear) and an antiserum against 17α-hydroxyprogesterone-3-oxime BSA (Teikoku Zoki Pharm. Co.). The antiserum cross-reacted with progesterone, 20α-hydroxyprogesterone, and pregnenolone at 7.85, 3.23, and 0.52%, respectively.

17α,20β-P was determined using [1,2,6,7-^{3}H] 17α,20β-P and an antiserum against 17α,20β-dihydroxy-4-pregnen-3-oxime-BSA which was kindly provided by Dr. Y. Nagahama, National Institute for Basic Biology, Okazaki, Japan. The antiserum to 17α,20β-P cross-reacted with 17α, 20β-P, 17α,20α-P, and 5β-pregnane-3β,17α,20β-triol at 2.54, 1.55, and 0.82%, respectively.

17α,20α-P was determined using [1,2,6,7-^{3}H] 17α,20α-P and an antiserum against 17α,20α-dihydroxy-4-pregnen-3-oxime-BSA which was kindly provided by Dr. A. Kambegawa, Department of Obstetrics and Gynecology, Teikyo University School of Medicine, Tokyo, Japan. The antiserum to 17α,20α-P cross-reacted with 17α,20β-P, progesterone, and deoxycortisol at 0.48, 0.17, and 0.10%, respectively.

Plasma levels of estradiol-17β were determined using [2,4,6,7-^{3}H] estradiol-17β (New England Nuclear) and an antiserum against estradiol-17β-6-CMO-BSA (Teikoku Zoki Pharm. Co.). The antiserum against estradiol-17β cross-reacted with estrone, estriol, and testosterone at 3.2, 1.77, and 0.29%, respectively.

Validation of the system for use in walking catfish plasma was achieved by obtaining parallel curves for serial dilutions of plasma samples collected from several fishes. Intraassay and interassay coefficients of variation at binding rates of 25%, 50% and 75% are presented in Table 1.

EIA

Plasma 20β-S levels were measured using a specific EIA. The method for steroid extraction for EIA was the same as that for RIA. Samples were reconstituted with 0.05% borate buffer containing 0.5% BSA (pH 7.8). An antiserum was raised against 17α,20β,21-trihydroxy-4-pregnen-3-CMO-BSA. This antiserum cross-reacted with progesterone, 17α-P, 17α,20β-P, and 17α,20α-P at 1.0, 0.01, 0.01 and 0.8%, respectively. Horseradish peroxidase (Sigma, USA) was used for labeling the antigen. Absorbance at 492 nm was measured using an EIA reader (Bio Rad, England) for microtiter plates. Intraassay and interassay coefficients of variation at binding rates of 25%, 50% and 75% are presented in Table 1.

TABLE 1. Intraassay and interassay coefficients of variation measured at 25, 50, and 75% of binding rate, respectively

Steroids	Intraassay			Interassay		
	25%	50%	75%	25%	50%	75%
Testosterone	19.6	6.6	6.0	22.6	11.9	18.0
Progesterone	4.0	5.1	8.3	4.7	8.1	4.3
17α-P	5.3	6.0	7.1	15.4	12.2	13.0
20β-S	13.0	12.5	11.2	19.5	17.4	12.6
17α, 20α-P	9.7	6.8	4.0	10.2	8.7	11.0
17α, 20β-P	3.3	4.7	9.7	5.0	9.3	9.0
Estradiol-17β	10.9	4.8	3.0	7.2	5.5	8.6

Details for this EIA will be published separately (Asahina *et al.*, unpublished data).

Statistics

The Student-t test was used to compare means between treated and control groups. The multiple range test of Duncan and the Kruskal-Wallis test were used to analyze the time course changes in each group.

RESULTS

Ovulation occurred in all fish in the HCG-treated group. Of 14 fish treated with HCG, 5 fish ovulated at 20 hr and 9 fish ovulated at 24 hr following treatment. GVBD was observed at 12 and 16 hr. However, all fish in the control group failed to ovulate.

Changes in plasma testosterone levels both in the HCG-treated and control group are shown in Fig. 1. In the HCG-treated fish, plasma testosterone levels showed a rapid increase ($P<0.01$; 0 hr vs 4 hr), peaking at 4 hr (38.3 ng/ml), and gradually returned to initial levels at 24 hr ($P<0.01$; 4 hr vs 24 hr). Plasma testosterone levels in the control group started to decrease at 16 hr ($P<0.01$; 0 hr vs 16 hr) without returning to initial levels at the end of the experimental period ($P<0.01$; 0 hr vs 52 hr).

Changes in plasma progesterone levels both in the HCG-treated and control groups are presented in Fig. 2. The change of amplitude in levels of this hormone was small but could be considered significant ($P<0.01$; 0 hr vs 12 hr). Progesterone levels in HCG-treated fish started to increase at 4 hr after the treatment ($P<0.01$; 0 hr vs 4 hr) and reached a peak (1.7 ng/ml) at 12 hr, and subsequently decreased below the detectable limit at 20 hr. With the exception of the beginning and at the end of the experimental period, plasma progesterone levels in the control group were always below the detectable limit.

Changes in plasma 17α-P levels both in the HCG-treated and control groups are shown in Fig. 3. Plasma 17α-P levels in the HCG-treated group increased at 8 hr ($P<0.01$; 0 hr vs 8 hr), peaked at 12 hr (20.0 ng/ml) ($P<0.01$; 8 hr vs 12 hr), and then rapidly returned to initial levels after 16 hr ($P<0.01$; 12 hr vs 16 hr). Thereafter, hormone levels showed small fluctuations. No significant change was observed in the control group.

Changes in plasma 17α,20β-P levels both in the

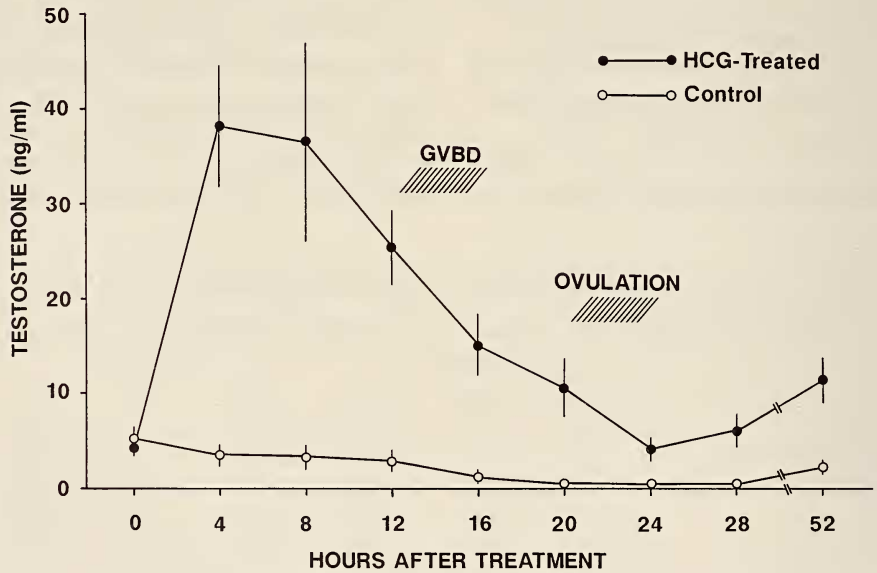

Fig. 1. Changes in plasma testosterone levels during HCG-induced ovulation in walking catfish. Data from 4–20 hr represent 7 and 5 fish for treated and control groups, respectively. Subsequent data represent 14 and 10 fish for treated and control groups, respectively. Each point is represented as mean $\pm$ SEM.

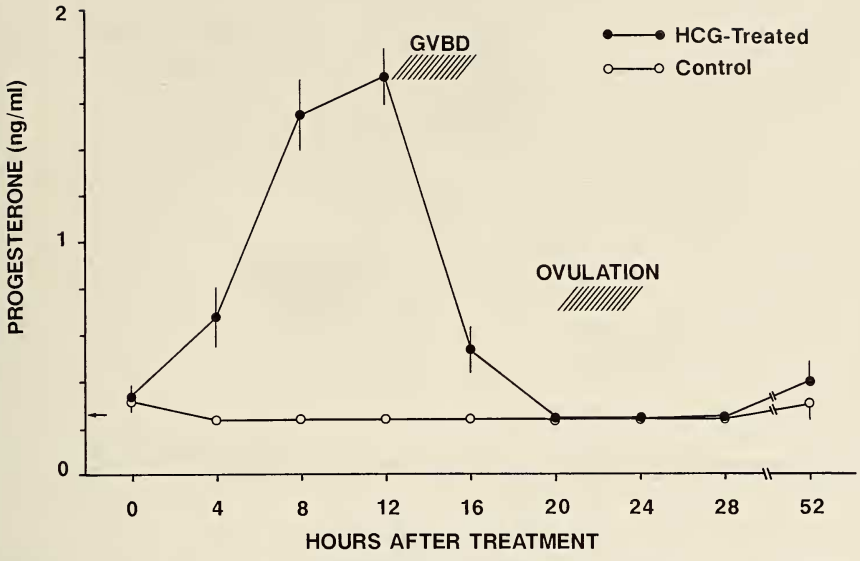

FIG. 2. Changes in plasma progesterone levels during HCG-induced ovulation in walking catfish. Arrow indicates the detectable limit of the assay. See Fig. 1 for experimental detail.

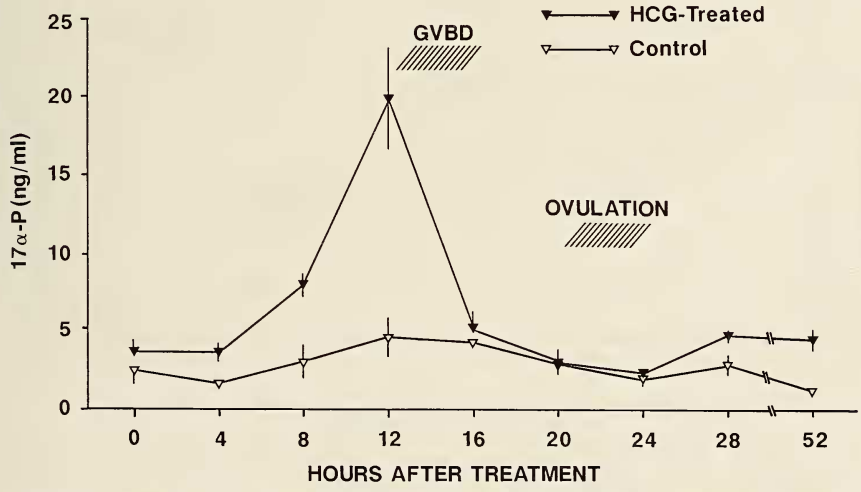

FIG. 3. Changes in plasma 17α-P levels during HCG-induced ovulation in walking catfish. See Fig. 1 for experimental detail.

HCG treated and control group are shown in Fig. 4. In the HCG-treated fish, plasma 17α,20β-P levels were below the detectable limit of 0.24 ng/ml until 8 hr after injection followed by a sudden increase ($P<0.01$; 8 hr vs 12 hr) until reaching a peak (8.3 ng/ml) at 12 hr in association with the initiation of GVBD. Thereafter, the levels dropped below the detectable limit at 24 hr ($P<0.01$; 12 hr vs 24 hr). Plasma 17α,20β-P levels of the control group were always below the detectable limit during the course of the experiment.

Changes in plasma 20β-S levels both in the HCG treated and control group are shown in Fig. 5. As just observed in 17α,20β-P levels, plasma 20β-S

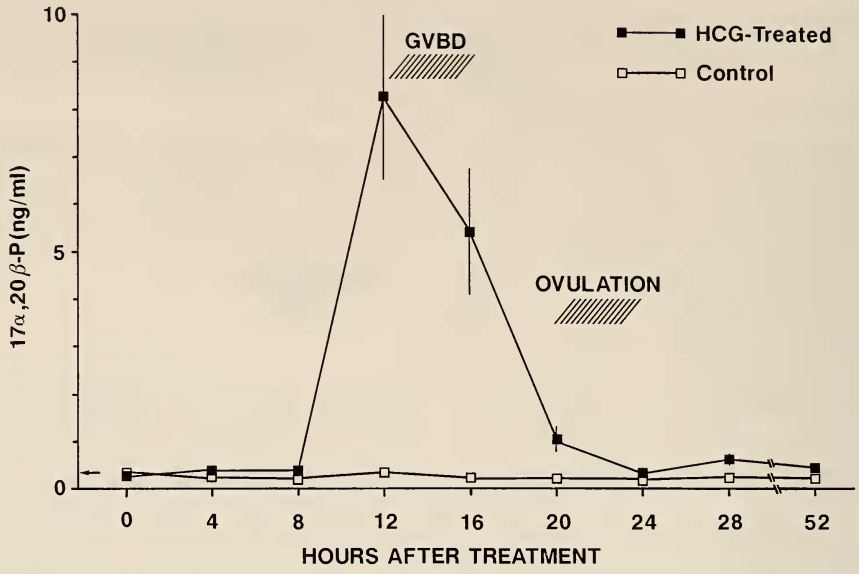

Fig. 4. Changes in plasma 17α, 20β-P levels during HCG-induced ovulation in walking catfish. Arrow indicates the detectable limit of the assay. See Fig. 1 for experimental detail.

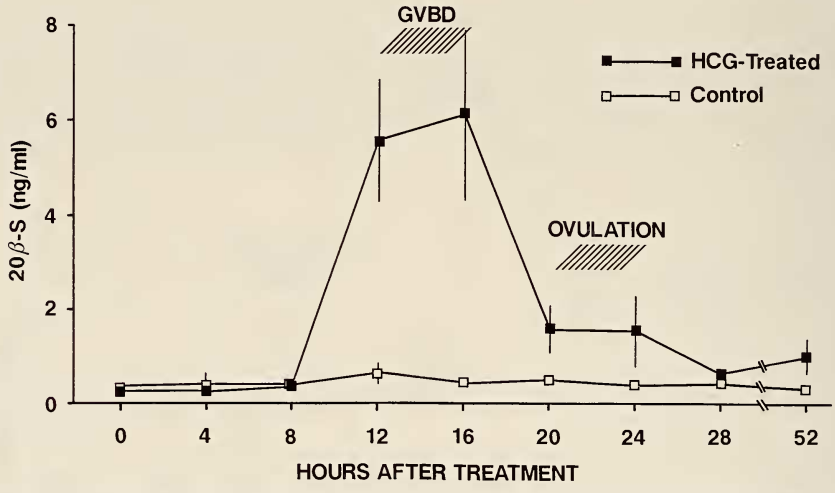

Fig. 5. Changes in plasma 20β-S levels during HCG-induced ovulation in walking catfish. See Fig. 1 for experimental detail.

levels stayed low during 8 hr after injection, then increased rapidly at 12 hr (5.6 ng/ml; $P<0.01$; 8 hr vs 12 hr). Levels ramained high until 16 hr and then decreased returning to the initial levels threafter. On the other hand, no significant changes occurred in the control group during the course of the experiments.

Changes in plasma 17α,20α-P levels both in the HCG treated and control group are shown in Fig. 6. The hormone started to increase at 4 hr ($P<$ 0.01; 4 hr vs 12 hr), peaked at 12 hr (2.2 ng/ml), and then decreased. The magnitude of its peak was lower than those of 17α,20β-P or 20β-S. Plasma 17α,20α-P in the control group remained under detectable limits throughout the experiments.

Changes in plasma estradiol-17β levels both in

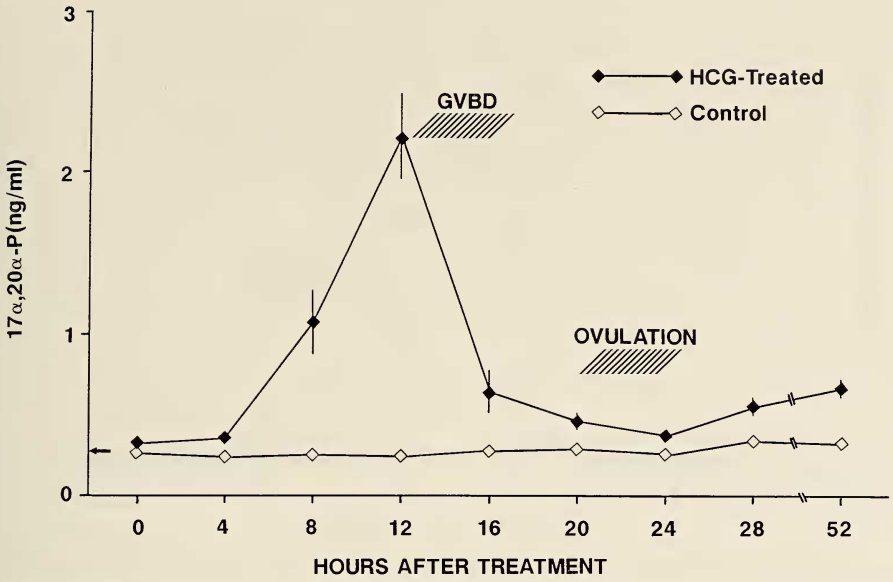

FIG. 6. Changes in plasma 17α, 20α-P levels during HCG-induced ovulation in walking catfish. Arrow indicates the detectable limit of the assay. See Fig. 1 for experimental detail.

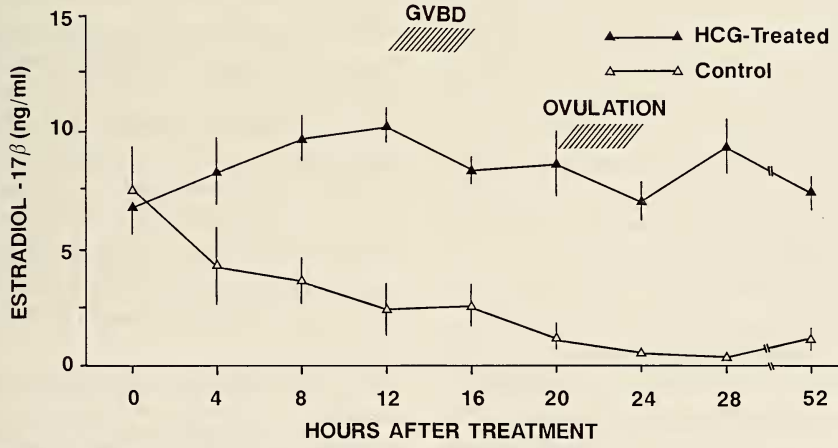

FIG. 7. Changes in plasma estradiol-17β levels during HCG-induced ovulation in walking catfish. See Fig. 1 for experimental detail.

the HCG-treated and control groups are presented in Fig. 7. In the HCG-treated group, no singificant change was observed, and plasma levels were maintained at relatively high levels. In the control group, however, a gradual decrease occurred during the experiment ($P<0.01$; 0 hr vs 20 hr).

Changes in the seven sex steroid hormone levels during ovulation in the HCG-treated female walking catfish are summarized in Fig. 8.

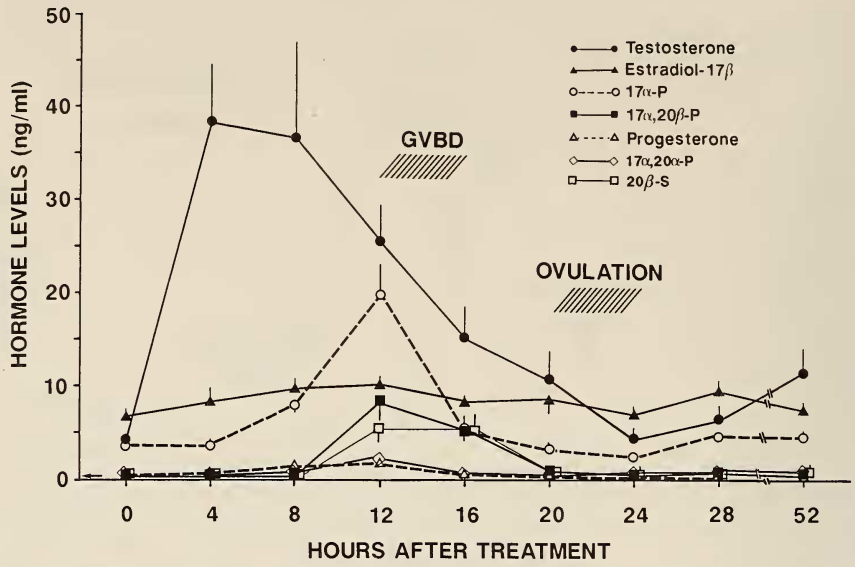

Fig. 8. Changes in the seven sex steroid hormone levels during HCG-induced ovulation in walking catfish. Arrow indicates the detectable limit of the assay. See Fig. 1 for experimental detail.

DISCUSSION

HCG has been used effectively in *Clarias macrocephalus* [11] and *Clarias lazera* [12] in the induction of ovulation. Recently, HCG has been successfully employed in inducing normal ovulation of *Clarias batrachus* [6]. In the present experiment, of 14 fish treated with HCG, 5 fish ovulated at 20 hr and 9 fish ovulated at 24 hr. These results indicate that the function of internal GtH in catfish can be replaced by HCG.

Various doses of HCG (0.2, 0.4, 0.8, and 1.6 IU/g body weight) have been tried in inducing ovulation of walking catfish (Zairin *et al.*, unpublished data). In all the trials, HCG treatment succeeded in inducing ovulation at dosages of 0.4 IU/g body weight or higher. At 0.4 IU/g body weight, the quantity of ovulated eggs was small and practically negligible, whereas a large number of ovulated eggs were observed in the group treated with 0.8 and 1.6 IU HCG/g body weight. Detail about the experiment above will be reported in the next paper. Considering that there is no significant difference between dosages of 0.8 and 1.6 IU/g body weight, dose of 0.8 IU HCG/g body weight has been chosen in the present experiment.

Testosterone levels peaked at 4 hr. Among the sex steroids monitored, this peak is the earliest and highest. This suggests that the injection of HCG activates steroidogenesis in the follicular layer. It is commonly accepted at present that GtH works on the theca cells of the follicle and stimulates the synthesis of testosterone which in turn is converted into estradiol-17β in granulosa layer [13]. In contrast, testosterone levels in the control groups decreased during the experiment. This decrease is probably due to some unknown problems in the sampling protocol, such as repeated blood sampling, stress caused by handling or an effect of anesthetics.

HCG injection caused testosterone to peak at 4 hr which was followed by the increase in 17α-P, a precursor of 17α,20β-P. This suggests that the shift in the steroidogenic pathway, from androgen to progestin synthesis, is induced by HCG injection as is proposed according to *in vitro* studies in *Clarias macrocephalus* [14]. 17α-P secreted from thecal layer is likely converted into 17α,20β-P in the granulosa layer where HCG, an external GtH, acts to enhance the activity of 20β-HSD, which is a key enzyme in this conversion [1]. As a result, a peak of 17α,20β-P occurs and propels the oocytes to final maturation.

Progesterone levels in this experiment increased over the basal level. It is not known whether this hormone possesses any role in oocyte maturation and ovulation in fishes. *In vitro* studies in *Clarias lazera* [15], however, showed that no changes in progesterone levels occurred at and after ovulation. Most likely, progesterone is not involved in either final maturation or ovulation.

In this experiment, 17α-P levels, a precursor of 17α,20β-P, increased prior to those of 17α,20β-P, showing their precursor-product relationship. Both of these hormones showed characteristic changes: very high levels during the oocyte maturation process, declining levels at ovulation. In unovulated fish of the same species, 17α-P was detected in low levels throughout the year (Zairin *et al.*, unpublished data), whereas 17α,20β-P was under detectable limit of our assay. Accordingly, these changes strongly suggest that both steroids play important roles in oocyte maturation in this species. The occurrence of prominent peak in both hormones around the time of oocyte maturation and ovulation has been reported in goldfish [16], bitterling [7], carp [17], and salmonids [18–19]. The pattern of increase in both 17α,20β-P and 17α-P seems to differ according to species. In bitterling, 17α,20β-P increased over 17α-P at peak [7]. However, in the present investigation, 17α-P increased over 17α,20β-P. Similar results were obtained in the winter flounder, *Pseudopleuronectes americanus* [20].

In the present study, we found that the plasma levels of 20β-S also increased during several hours prior to ovulation. Since 20β-S is as effective as 17α,20β-P in inducing final oocyte maturation in some teleost species [2], it is probable that both 17α,20β-P and 20β-S act as MIS in this species. Some *in vitro* experiments are being undertaken to ascertain this.

The status of the maturation inducing steroid in catfish has been a source of controversy for quite a while. Formerly, corticosteroid was regarded as an oocyte maturation inducing substance in an Indian catfish, *Heteropneustes fossilis* [21–23]. However, subsequent results from *in vitro* studies on the same species [24] did not support this hypothesis. It was later reported that MIS in another Indian catfish, *Mystus vittatus* is 17α,20β-P [25]. As mentioned previously, both 17α,20β-P and 20β-S levels increased prior to ovulation in walking catfish. This result seems to lead to the so-called "multiple MIS" theory in teleosts. This also suggests the existence of a correlation between the ovary and interrenal kidney as shown in old maturation theories of catfish, because 20β-S is a form of corticoid. On the other hand, although 17α,20α-P also increased prior to ovulation, the role of this steroid, the isomer of 17α,20β-P, is of yet uncertain, since the activity of 20α-HSD is very low compared to that of 20β-HSD both *in vitro* and *in vivo*.

Patterns of fluctuation in estradiol-17β in this experiment are particularly interesting, as this hormone did not peak following a peak of testosterone. HCG injection did not change the plasma estradiol-17β levels. This is in contrast to goldfish [26], where a peak of estradiol-17β is subsequent to a peak of testosterone. Estradiol-17β levels in the control groups decreased during the experiment, and did not return to the basal levels as expected, likely due to some unknown problems in the sampling protocol, such as repeated blood sampling, stress caused by handling or an effect of anesthetics. Considering this possibility, it could be assumed that estradiol-17β production in the HCG-treated group is actually stimulated during ovulation. In another experiment, when spawning is induced experimentally by water and temperature level manipulation, the occurrence of high levels of estradiol-17β in ovulated- and just-spawned females were observed (Zairin *et al.*, unpublished data). In the present experiment, higher estradiol-17β levels in the HCG-treated group than in the control group may be due to the action of HCG on the vitellogenic oocytes, as this fish possesses various sizes of oocytes throughout the year (Zairin *et al.*, unpublished data).

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